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Caracterização genética de isolados de *Fusarium oxysporum* f. sp. *cubense* e desenvolvimento de um marcador SCAR para identificação de suscetibilidade ao mal-do-panamá da bananeira

Tese submetida ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do Grau de Doutora em Ciências.

Orientador: Prof. Dr. Marciel João Stadnik

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por

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*“Eu sei como ele conseguiu.  
Todos perguntaram: Pode nos dizer como?  
É simples, respondeu o Einstein.  
Não havia ninguém ao seu redor, para lhe dizer  
que não seria capaz”.*

*Albert Einstein*



**Dedico**

***“A Deus, a minha mãe e ao meu amado esposo esta conquista”***



## RESUMO

O mal-do-panamá, causado por *Fusarium oxysporum* f. sp. *cubense* (*Foc*), é uma doença endêmica em todas as regiões produtoras de banana, sendo citado como uma das seis doenças mais importantes de plantas cultivadas e responsável por grandes perdas econômicas. O cultivo da bananeira em Santa Catarina sofre com essa doença e uma das estratégias usadas no seu controle é a substituição das cultivares do subgrupo Prata, consideradas suscetíveis ao fungo, pelas cultivares do subgrupo Cavendish, devido a sua maior resistência. Nos últimos anos, no entanto, essa estratégia vem sendo questionada em função da ocorrência de focos de *Foc* nos bananais de cultivares do subgrupo Cavendish. Tendo em vista a importância da bananicultura no Sul do Brasil, região subtropical, a constatação do avanço da doença e o aumento de focos do mal-do-panamá em bananais de cultivares do subgrupo Cavendish nessa região, este trabalho teve como objetivo caracterizar isolados de *Fusarium oxysporum* f. sp. *cubense* coletados de cultivares de bananeira dos subgrupos Prata e Cavendish e desenvolver um marcador SCAR associado à suscetibilidade ao mal-do-panamá. Avaliou-se uma população de 152 isolados de *Foc* coletados em 13 regiões produtoras de banana do Estado de Santa Catarina por meio de características morfológicas, níveis de agressividade, utilização de marcadores moleculares microssatélites e intermicrossatélites (SSR, ISSR) e também pelo sequenciamento do fator de alongação TEF 1 $\alpha$ . A patogenicidade foi confirmada para todos os isolados, e os critérios morfológicos confirmaram a identidade do fungo. Em relação aos níveis de agressividade, foi possível classificar os isolados em três grupos distintos, considerados como pouco agressivo moderadamente agressivo e agressivo. Quando utilizamos as técnicas moleculares, observou-se que os resultados de ISSR, SSR e sequenciamento TEF 1 $\alpha$  revelaram uma menor variação entre os isolados analisados, sendo que, a maioria apresentou 100% de similaridade interna. Entre esses marcadores, ISSR + SSR foi o mais polimórfico, sendo capaz de diferenciar mais indivíduos e com uma maior percentagem de dissimilaridade. Na segunda etapa desse trabalho, aplicando-se 78 iniciadores arbitrários em DNA genômico de genótipos resistentes e suscetíveis, uma banda de RAPD associada à susceptibilidade foi selecionada e usada para gerar o iniciador SCAR SuscPD-F/SuscPD-R. Este marcador foi validado em 28 cultivares de bananeira e os resultados indicaram um elevado grau de especificidade do marcador, o qual foi capaz de discriminar entre os dois grupos de contraste resistentes ou suscetíveis com um poder

discriminatório de 93%. Os resultados obtidos poderão apoiar os trabalhos de melhoramento genético da cultura, acelerando a seleção e o desenvolvimento de cultivares com maior nível intrínseco de resistência às infecções e melhorias nas técnicas de controle à doença. Esse trabalho representa uma contribuição na caracterização de *Foc* e no estudo de resistência da bananeira em áreas subtropicais, onde há até o momento uma carência de resultados e ferramentas nesta área.

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## 1 INTRODUÇÃO E JUSTIFICATIVA

A banana (*Musa spp*) é uma das frutas mais consumidas no mundo, sendo cultivada na maioria dos países tropicais. Esta fruta ocupa a segunda posição na produção mundial de frutas e juntamente com o arroz, o trigo e o milho são consideradas como as fontes alimentares mais importantes do mundo. Devido ao seu alto valor nutritivo e por estar disponível durante todo o ano, é uma fruta de suma importância econômica e social. No Brasil a banana é uma das frutas mais cultivadas, apresentando uma produção crescente desde 2003. As condições de clima (temperatura, umidade relativa, precipitação e insolação) favorecem a constante produção anual, atendendo de forma regular as necessidades de consumo (Vieira, 2013).

O estado de Santa Catarina tem uma área cultivada de aproximadamente 29,2 mil hectares e uma produção anual de 689 mil toneladas, o que corresponde a um rendimento médio de 23,5 toneladas por hectare. Estima-se que no estado o seu cultivo seja explorado por aproximadamente 25 mil produtores rurais, sendo que seis mil tem na bananicultura sua principal fonte de renda. Santa Catarina destaca-se no cenário nacional como o terceiro maior produtor de banana. O litoral norte do estado de Santa Catarina concentra 85% da produção, onde predominam os cultivares Nanica e Nanicão, componentes do tipo Caturra, pertencentes ao subgrupo Cavendish. Já no litoral sul, que representa cerca de 9% da produção, os cultivares mais usados são a Enxerto e a Branca de Santa Catarina, componentes do tipo Prata pertencentes ao subgrupo Prata (Vieira, 2013).

Apesar desses fatores positivos, das novas tecnologias de manejo e da disponibilidade de novas cultivares resistentes às doenças, a bananicultura vem acumulando perdas econômicas nos últimos anos principalmente por problemas fitossanitários que também implicam o aumento dos custos de produção. As doenças fúngicas constituem os principais problemas fitopatológicos da bananeira, normalmente afetada em todo o seu ciclo vegetativo e produtivo, nas suas diversas partes (raízes, pseudocaule, folhas e frutos). Esses patógenos, em alguns casos, são fatores limitantes da produção dessa fruta (Ventura & Hinz, 2002).

Entre os principais problemas fitossanitários da bananeira, destaca-se o mal-do-panamá, causado pelo fungo *Fusarium oxysporum* f. sp. *cubense* (*Foc*), que ocorre principalmente nos bananais das cultivares do subgrupo Prata, e ultimamente vem atacando as cultivares do subgrupo Cavendish. A doença causa a morte de plantas, e dificulta e

até mesmo impede a implantação de novos plantios, pois o fungo pode sobreviver no solo durante muitos anos (Cordeiro et al., 2005). Como o uso de fungicidas não é eficiente, o uso de cultivares resistente é a forma de controle. Porém, a eficiência depende tanto da resistência dos materiais genéticos disponíveis como da variabilidade genética dos patótipos do patógeno. Portanto, é importante conhecer as raças e/ou patótipos predominantes em um determinado local e sua epidemiologia, para contribuir com os programas de melhoramento.

Técnicas de biologia molecular e análise das regiões rDNA-ITS e TEF têm contribuído para os estudos de variabilidade de *Foc* (Bogale et al., 2005; Groenewald et al., 2006; Leong et al., 2009, Leong et al., 2010). Marcadores moleculares diferenciam-se pela tecnologia utilizada para revelar variabilidade em nível de DNA, e assim variam quanto à habilidade de detectar diferenças entre indivíduos, custo, facilidade de uso, consistência e repetibilidade (Ferreira & Grattapaglia, 1998). Neste sentido a associação da biologia molecular com os estudos clássicos de fitopatologia contribuirá para a otimização na seleção e no desenvolvimento de novas cultivares buscando, preferencialmente, maior produtividade, qualidade e resistência a doenças. Contribuirá também para a Produção Integrada de bananas, com ganhos ambientais resultantes do melhor conhecimento da epidemiologia das pragas e técnicas de manejo e controle integrado.

O objetivo deste trabalho foi caracterizar morfológicamente e geneticamente o fungo *Fusarium oxysporum* f. sp. *cubense* isolados de bananeiras dos subgrupos Prata e Cavendish coletados em diferentes municípios do estado de Santa Catarina, que foi contemplado no Capítulo 1 deste trabalho e, desenvolver um marcador molecular SCAR associado à suscetibilidade ao mal-do-panamá, incluído no Capítulo 2. Os dois capítulos foram escritos na forma de artigo e submetidos a periódicos científicos.

## 2 OBJETIVOS

### 2.1 OBJETIVOS GERAIS

Caracterizar o fungo *Fusarium oxysporum* f. sp. *cubense* (*Foc*) isolado de cultivares de bananeira pertencentes aos subgrupos Prata e Cavendish, provenientes dos municípios produtores de banana do estado de Santa Catarina, por meio de descritores morfológicos e moleculares e desenvolver marcador SCAR capaz de diferenciar cultivares de bananeira resistente ao *Foc*.

### 2.2 OBJETIVOS ESPECÍFICOS

- Identificar baseados em marcadores morfológicos isolados de *Fusarium oxysporum* f. sp. *cubense* obtidos de cultivares de bananeiras dos subgrupos Prata e Cavendish;
- Testar a patogenicidade e avaliar a agressividade dos isolados de *Fusarium oxysporum* f. sp. *cubense* em cultivares dos subgrupos Prata e Cavendish;
- Estudar a variabilidade genética dos isolados de *Fusarium oxysporum* f. sp. *cubense* utilizando a técnica de marcadores moleculares SSR e ISSR;
- Sequenciar a região TEF-1α dos isolados de *Fusarium oxysporum* f. sp. *cubense* e comparar com as sequências depositadas no Genbank.
- Identificar por meio de marcadores RAPD, uma marca específica que possa ser convertida em um marcador SCAR, capaz de diferenciar cultivares dos subgrupos Prata e Cavendish resistentes ao *Fusarium oxysporum* f. sp. *cubense*.



### 3 REVISÃO BIBLIOGRÁFICA

#### 3.1 A BANANICULTURA

As bananeiras são plantas da classe das Monocotiledôneas, ordem Scitaminales, família Musaceae. O centro de origem da maior parte do germoplasma de bananeira está localizado no continente asiático, ocorrendo centros secundários na África Oriental, algumas ilhas do Pacífico e uma considerável diversidade genética na África Ocidental. A planta se caracteriza por apresentar caule suculento e subterrâneo (rizoma), cujo "falso" tronco (pseudocaule) é formado pela sobreposição das bainhas das folhas, folhas grandes e flores, em cachos que surgem em série a partir do chamado "coração" da bananeira. É uma planta tipicamente tropical, exige calor constante, precipitações bem distribuídas e elevada umidade para o seu bom desenvolvimento e produção. Sua altura pode variar de 1,8m a 8,0m. Dada a característica de emitir sempre novos rebentos, o bananal é permanente na área, porém com as plantas se renovando ciclicamente. A banana está entre as frutas mais consumidas no mundo e no Brasil, sendo um alimento energético, rico em carboidratos, sais minerais, como sódio, magnésio, fósforo e, especialmente, potássio. Apresenta predominância de vitamina A e C, contendo também as vitaminas B1, B2 e B6, contém pouca proteína e gordura (CROUCH, 1999).

Nos últimos anos, registrou um aumento significativo no volume produzido, com 106,542 milhões de toneladas. A banana é a segunda fruta mais consumida no mundo, com 11,4 kg/hab/ano. Perde apenas para a laranja, com 12,2 kg/hab/ano e segundo a FAO, cresce a cada ano, graças ao empenho do setor produtivo na qualificação da produção e do setor mercadológico nos aspectos que envolvem a apresentação do produto, a vida útil de prateleira, e a divulgação dos benefícios para quem o consome.

Em Santa Catarina a bananicultura é uma das atividades agrícolas de grande importância social e na geração de empregos. Em termos sociais, é explorada por cerca de 25 mil produtores rurais, sendo que seis mil têm na bananicultura sua principal atividade de renda. O Estado catarinense é o terceiro maior produtor de bananas do Brasil, ocupando a maior área cultivada de fruteiras do Estado, com 29,2 mil hectares e uma produção anual de 689 mil toneladas. No Estado, as maiores produções por município se concentram no município de Corupá, responsável por 20%, seguido por Luiz Alves, com 19%, Massaranduba, com 9%, Jacinto Machado, com 7% e Jaraguá do Sul, com 6%. A produtividade

média dos pomares catarinense tem sido sempre crescente, fazendo com que o Estado continue sendo referência nacional nessa cultura. (Vieira 2013).

### 3.2 A DOENÇA MAL-DO-PANAMÁ

O mal-do-panamá, ou murcha de *Fusarium*, é uma doença endêmica em todas as regiões produtoras de banana do mundo, sendo citada como uma das seis mais importantes doenças de plantas cultivadas e responsável por perdas econômicas na ordem de bilhões de dólares (PLOETZ, 2006).

A primeira constatação da doença foi em 1876, na Austrália, seguindo-se relatos de ocorrência da doença no Panamá e na Costa Rica em 1890. Posteriormente, foi disseminada, para todos os países da América Central e do Sul (STOVER, 1972).

Existem duas hipóteses em relação à origem do fungo. A primeira é que o fungo teria surgido na Ásia e se dispersado para a África e Américas através do transporte de rizomas e/ou plantas infectadas. Na segunda hipótese, o fungo teria se originado em várias regiões e co-evoluído independentemente. Bentley et al. (1998) sugerem que o isolamento genético e a distribuição geográfica limitada de diferentes linhagens seja um indício de que, provavelmente, elas tenham se desenvolvido independentemente dentro e fora do centro de origem do hospedeiro. A ocorrência mundial do fungo, por enquanto, está localizada de 30° N a 30° S, não havendo ainda registros nas Ilhas do Pacífico Sul, Somália e Mediterrâneo (PEREZ; VICENTE, 2004; PLOETZ, 2006).

Sua primeira constatação no Brasil foi em 1930, no município de Piracicaba, São Paulo, na cultivar Maçã. Em apenas 3-4 anos foram dizimados cerca de um milhão de plantas de banana naquele município paulista (GOES; MORETO, 2001).

### 3.3 ETIOLOGIA

O agente etiológico do mal-do-panamá é o fungo *Fusarium oxysporum* f. sp. *cubense*, não se conhecendo seu estágio sexuado. De um modo geral, as *formae speciales* de *Fusarium oxysporum* não podem ser distinguidas morfológicamente. Devido à plasticidade e variações de características fenotípicas encontradas neste fungo, a taxonomia baseada somente em conceitos morfológicos não é confiável (Leslie & Summerell, 2006). As colônias crescem 4 a 7mm.dia<sup>-1</sup> sobre agar batata



dextrose a 24°C, com abundante micélio aéreo, com coloração que vai do branco ao violeta (Ploetz, 2006). As duas principais formas de esporos de *Fusarium* são os microconídios e os macroconídios. Os microconídios são unicelulares ovais a reniformes e hialinos e uninucleados; os macroconídios são mais comuns sendo fusiformes, falcados multicelulares, mas cada célula tem somente um núcleo. Todos os núcleos de um macroconídio, contudo, são descendentes mitóticos de um mesmo núcleo progenitor e são, portanto, geneticamente idênticos (PUHALLA, 1985).

O patógeno pode permanecer no solo em estágio de dormência por vários anos, na forma de clamidósporos. Quando estimulados, germinam, desenvolvendo micélio. A partir desses clamidósporos, são produzidos dentro de poucas horas, conídios e outros clamidósporos, novamente após dois a três dias (STOVER, 1972). Os clamidósporos são estruturas de sobrevivência do fungo e são formados nos tecidos necrosados e no solo.

Inúmeros fatores, incluindo a perda da distinção clara das espécies através de características morfológicas, levam a conceitos que são amplos e juntamente com a variação e mutação dentro da cultura, têm conspirado para criar um sistema taxonômico que não reflete a diversidade das espécies. Isto tem gerado resultados controversos na aplicação de nomes de espécies para isolados patogênicos e toxigênicos (GEISER et al., 2004). O gênero ainda apresenta uma série de variações de características morfológicas e patogênicas, resultando em uma classificação complexa dividida em seções, *formae speciales* e raças. O conceito *formae speciales* foi aplicado por Snyder & Hansen (1953) para reconhecer isolados patogênicos que foram morfológicamente semelhantes a isolados saprofíticos de mesma espécie, mas que se diferenciam em sua habilidade de causar doença em hospedeiros específicos. Isolados patogênicos de *Fusarium oxysporum* estão classificados em mais de 120 *formae speciales* e raças.

Em *Fusarium oxysporum* f. sp. *cubense* são conhecidas quatro raças fisiológicas do patógeno, sendo que as raças 1, 2 e 4 são importantes à bananeira. A raça 3 ocorre apenas em *Heliconia* sp. No Brasil, de acordo com a estrutura dos grupos de compatibilidade vegetativa dos isolados de *Fusarium oxysporum* f. sp. *cubense* analisados, presume-se a prevalência da raça 1 (Goes & Moretto, 2001). A forma mais simples de diferenciação das raças seria mediante o uso de variedades indicadoras, onde a variedade Gros Michel é indicadora da raça 1, a Bluggoe, indicadora da raça 2 e as variedades do subgrupo Cavendish (Nanica, Nanicão e Grande Naine) são indicadoras da raça 4

(Cordeiro et al., 2005).

Recentemente foi reportada a ocorrência de um novo biotipo da Raça 4, a Raça Tropical 4 ou TR4, que está devastando plantações comerciais de banana em Taiwan, Malásia, Sumatra, Sulawesi, Filipinas, Vietnam, China e Austrália. Assim, foram designados dois tipos de raça 4, raça subtropical 4 (ST4) e raça tropical 4 (TR4). No entanto, enquanto ST4 isolada causa doença em Cavendish nos subtrópicos, principalmente quando as plantas estão expostas a estresses abióticos, a TR4 isolada é patogênica em ambas as condições, tropical e subtropical (Buddenhagen, 2009). A disseminação da TR4 para os países produtores de banana dos continentes africano e americano representa uma séria ameaça para a bananicultura mundial, pois além do subgrupo Cavendish, afeta as cultivares que geram 80% da produção mundial de bananas (Ploetz, 2009). No entanto, considerando-se a hipótese de Bentley et al. (1998) que sugere que o fungo teria se originado em várias regiões com grupos coevoluindo independentemente dentro e fora do centro de origem do hospedeiro, seria possível a ocorrência das raças ST4 e TR4 em outras regiões sem que necessariamente tenham sido disseminadas para essas regiões.

Baseado no fato de existirem no solo linhagens não patogênicas de *F. oxysporum*, é possível que a sobrevivência ocorra também em estado saprofítico. Esta hipótese pode ser reforçada pelo fato de que linhagens não patogênicas são capazes de formar heterocários com linhagens patogênicas. Assim, os núcleos da forma patogênica persistem no micélio de crescimento saprofítico, voltando a atuar quando em presença da planta hospedeira (Cordeiro & Matos, 2000).

A disseminação do patógeno pode ocorrer de diversas formas. Os rizomas, raízes e pseudocaules de plantas doentes liberam grande quantidade de inóculo na superfície do solo e a transmissão do patógeno depende do contato de raízes de plantas sadias com este inóculo. Outras formas freqüentes de disseminação são a água de irrigação, de drenagem e de inundação, animais, homem, equipamentos, ferramentas e material de plantio infectado (Ventura & Hinz, 2002). No Brasil, a disseminação via mudas infectadas assume grande importância, uma vez que são utilizadas em novos plantios. São do tipo convencional, sem os devidos cuidados na seleção do bananal fornecedor. Gradativamente, houve crescimento no uso de mudas micropropagadas que elimina essa via de disseminação (Cordeiro et al., 2005). A disseminação da doença para novas áreas está estritamente relacionada com a introdução de material propagativo suscetível (Stover, 1972).

### 3.4 SINTOMAS DA DOENÇA

As radículas e extremidades das raízes são os sítios iniciais de infecção. A infecção inicia-se nas radículas, atingindo o sistema vascular da bananeira, num processo sistêmico (Beckman, 1989). Nos genótipos resistentes, a infecção é paralisada devido à formação de géis (pectatos de cálcio e de magnésio) e tiloses nos vasos do xilema, enquanto nas cultivares suscetíveis, a colonização dos vasos continua, estendendo-se aos tecidos parenquimatosos anexos. Em estádios mais avançados da doença, ocorre a colonização do tecido parenquimatoso adjacente, com a produção de elevada quantidade de conídios e clamidósporos (Stover, 1972) Figura 1.

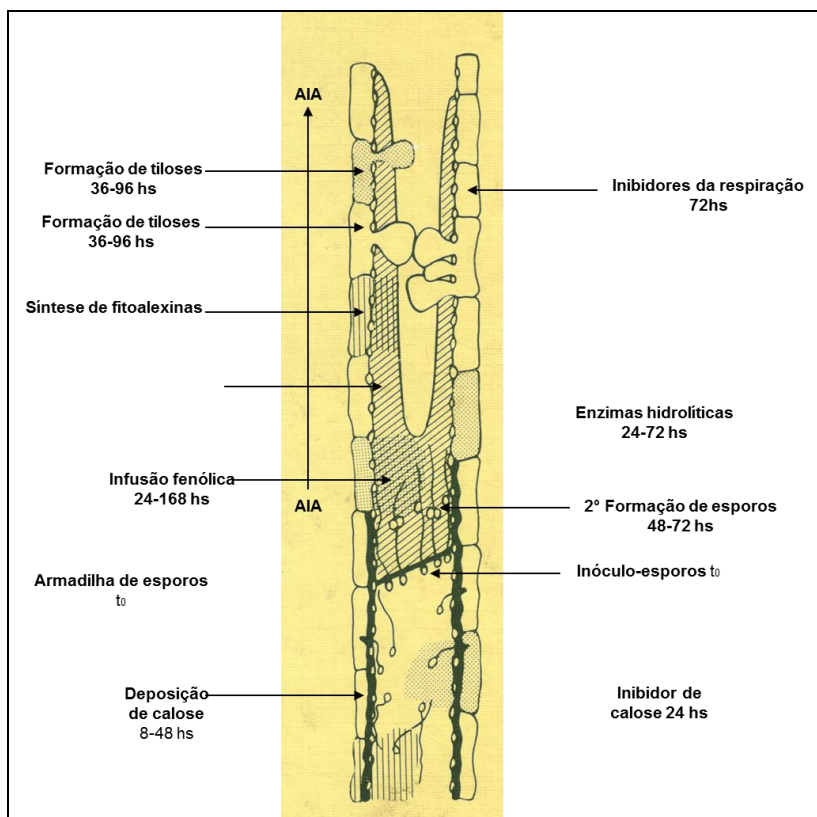


Figura 1 - Apresentação esquemática das interações entre *Fusarium oxysporum* e o hospedeiro dentro de um vaso condutor inicialmente infectados.

Os sintomas exibidos pelas plantas atacadas iniciam-se de 2 a 5 meses após a infecção e podem ser observados interna ou externamente nas folhas, no corte do rizoma e do pseudocaule Figura 2, (Codeiro et al., 2005).

De acordo com Ventura & Hinz (2002), externamente observa-se um amarelecimento progressivo, das folhas mais velhas para as folhas mais novas, começando pelos bordos do limbo em direção à nervura principal. Progressivamente ao amarelecimento, ocorre murcha, com posterior quebra do pecíolo junto ao pseudocaule, que dá à planta o aspecto típico de um guarda-chuva fechado. Além disto, pode ser observado estreitamento do limbo nas folhas mais novas, engrossamento das nervuras secundárias da folha e, ocasionalmente, necrose da folha vela. Um sintoma bastante típico e frequentemente encontrado é a rachadura do pseudo-caule, próximo ao solo, cujo tamanho varia com a área afetada no rizoma. Os sintomas são mais comuns em plantas adultas, mas podem ser encontrados também em plantas jovens.

Como sintomas internos observam-se pontuações pardo-avermelhadas e descoloração vascular ao se realizarem cortes transversais ou longitudinais no pseudocaule ou rizoma. As pontuações pardo-avermelhadas provavelmente surgem em função da oxidação de fenol quando na presença do patógeno. A descoloração vascular no pseudocaule concentra-se mais periféricamente, mantendo-se o centro claro. No rizoma, a descoloração é mais pronunciada na área de densa vascularização, onde o estelo se junta ao córtex, podendo-se observar a evolução dos sintomas do rizoma para as brotações a ele aderidas (Ventura & Hinz 2002; Cordeiro et al., 2005). O desenvolvimento do mal-do-panamá em banana está relacionado com a interação patógeno e genótipo da planta e parece ser fortemente influenciado pelas condições ambientais (Groenewald et al., 2006).

Em Santa Catarina o fungo ataca principalmente os bananais do subgrupo Prata, mas atualmente, o aumento de áreas de Cavendish apresentando sintomas desta doença tem causado inquietude a toda cadeia produtiva. A doença causa a morte de plantas e dificulta a implantação de novos plantios em locais infestados, porque o fungo permanece no solo durante muitos anos.

Esta doença não pode ser controlada através do uso de fungicidas. Em solos com baixa supressividade ao fungo, uma vez infestados, o cultivo de bananeiras suscetíveis à doença torna-se uma atividade de alto risco. Nessas condições, a única forma de permanecer na atividade é através da utilização de cultivares resistente (Ventura & Hinz, 2002).



Figura 2 - Sintomas da doença causado pelo fungo *Fusarium oxysporum* f. sp. *cubense* em bananeiras. A e B – sintomas externos; C e D – sintomas internos. A) Amarelecimento progressivo das folhas; B) Rachadura das bainhas no pseudocaule; C) pontuações pardo-avermelhadas no rizoma; D) necrose no tecido vascular do pseudocaule.

A busca por variedades resistentes, seja pela seleção de recursos genéticos existentes, ou pela geração de novas variedades por hibridação, é uma das principais linhas de ação, visando o controle desta doença. Um dos grandes obstáculos para a manutenção da resistência da

bananeira reside na variabilidade do patógeno, dessa forma torna-se importante e fundamental conhecer suas raças e/ou patótipos predominantes em um determinado local ou região, e sua epidemiologia, para subsidiar os programas de melhoramento e também contribuir no manejo e controle integrado (Silva & Bettiol, 2005).

### 3.5 ESTUDO MORFOLÓGICO E GENÉTICO *Fusarium oxysporum*

O estudo da diversidade populacional de *Foc* e o entendimento dos eventos moleculares no processo de diversificação são essenciais para o desenvolvimento de estratégias de manejo ao longo prazo. A análise e a quantificação de diversidade em fungos requer a combinação de ferramentas fenotípicas e genotípicas.

Os critérios morfológicos são o primeiro passo na identificação das espécies de fungos, mas podem ser complementados com a utilização de métodos mais acurados, como, por exemplo, os baseados nas características genéticas. As descrições para a classificação de *Fusarium oxysporum* têm base no uso de meios de cultura específicos e observações da cultura, quanto ao crescimento micelial, pigmentação. As estruturas de esporulação como: macroconídios, microconídios, formação de clamidosporos (ausentes ou presentes) e a taxa de crescimento, são critérios consistentes e são usados na identificação deste patógeno (Nelson et al., 1983; Leslie and Summerell 2006).

Técnicas de biologia molecular têm sido apresentadas para caracterizar fungos fitopatogênicos. A caracterização genética do *Fusarium oxysporum* têm sido estudada com a aplicação de marcadores moleculares (Viljoen et al., 2002; Groenewald et al., 2006; Fourie et al., 2009; Silva et al., 2010; Visser et al., 2010). Marcadores moleculares diferenciam-se pela tecnologia utilizada para revelar variabilidade em nível de DNA, e assim variam quanto à habilidade de detectar diferenças entre indivíduos, custo, facilidade de uso, consistência e repetibilidade (Ferreira & Grattapaglia, 1998). Alguns estudos baseiam-se na técnica molecular da reação em cadeia da polimerase (PCR) que é uma ferramenta robusta para diagnosticar e detectar fungos fitopatogênicos e tem contribuído grandemente para o manejo de doenças de plantas (Lanza, 2000; Karaoglu, 2005).

A técnica de microsatélite vem sendo utilizada para estudos de variabilidade genética de fungos (Silva, 2010; Dutech et al., 2007; Bogale et al., 2005). Os microsatélites ou seqüências simples repetida (SSR) são seqüências pequenas de nucleotídeos (1 a 6 bases) repetidas em *tandem*. Essas seqüências simples são bastante freqüentes e

distribuídas ao acaso no genoma dos eucariotos. Os loci SSR, se tornaram uma nova geração de marcadores genéticos, porém o uso deste marcador requer o conhecimento prévio da região que flanqueia o SSR para que se construam os iniciadores que serão usados para reação de PCR (Liu & Wendel, 2001).

Zietjewicz et al. (1994), desenvolveram um tipo de marcador baseado em SSR, as chamadas *Inter Simple Sequence Repeats*, (intersequência simples repetidas) ou simplesmente ISSR. Este marcador contorna a problemática da informação prévia das seqüências que flanqueiam os microssatélites. Em adição a análise de ISSR é tecnicamente simples em comparação a outros marcadores. Os marcadores ISSR são amplificados via PCR e não necessitam do sequenciamento da região, resultando ainda na obtenção de padrões altamente polimórficos (Thangavelu et al., 2012). Além disso, são dominantes, podendo gerar um grande número de alelos reproduzíveis e altamente polimórficos utilizando um iniciador complementar a um microssatélite alvo. Estes também foram escolhidos para evitar os problemas que os alelos nulos de microssatélites poderiam trazer para as análises desejadas, pois não requerem informações prévias de seqüências de DNA da espécie-alvo, produzem fragmentos com grande reprodutibilidade.

O gene codificador do fator de alongação (TEF) também é comumente utilizado como marcador filogenético para a diferenciação de espécies do gênero *Fusarium*, como mostra o trabalho que foi desenvolvido por O'Donnell, et al. (1998), no qual os autores utilizando esta técnica conseguiram verificar relações dentro do complexo de espécies *Fusarium oxysporum*. O gene TEF-1 $\alpha$  que codifica uma parte essencial do mecanismo de tradução de proteínas, tem uma utilidade filogenética alta. Este gene parece ser consistentemente de cópia única em *Fusarium* e mostra um nível elevado de polimorfismo de seqüências entre espécies intimamente relacionadas, mesmo em comparação com as porções ricas em introns de genes que codificam proteínas tais como: calmodulina,  $\beta$  tubulina e histona H3. TEF-1 $\alpha$  tem sido escolhido como uma ferramenta para identificação *Fusarium* (Nayaka et al., 2011).

Marcadores de DNA têm um grande potencial para melhorar a eficiência e precisão do melhoramento de plantas convencional e/ou seleção clonal por meio de seleção assistida por marcadores (Collard e Mackill 2008), em que a seleção dos indivíduos desejados é auxiliada pela utilização de marcadores moleculares. Sabe-se que a base fundamental do melhoramento de plantas é a seleção de plantas específicas com características desejáveis. O desenvolvimento de

técnicas moleculares permite a determinação de marcadores ligados a determinadas condições de importância econômica e constitui uma estratégia para a seleção rápida, confiável e reprodutível, acelerando o melhoramento através do conhecimento, nas fases iniciais, da reação a uma determinada característica e sua interação com o genótipo (Zambrano et al. 2007).

Uma estratégia utilizada para a identificação de marcadores moleculares relacionados a caracteres de interesse é a utilização de marcadores RAPD (amplificação aleatória do polimorfismo do DNA) que são ferramentas úteis em virtude da facilidade de utilização, rapidez, pouco custo (Javed et al., 2004). Para aumentar a confiabilidade desses marcadores e convertê-los em marcadores co-dominantes, Paran & Michelmore (1993) desenvolveram os marcadores SCAR (Região amplificada de sequência caracterizada).

Marcadores SCAR são derivados de marcas RAPD pelo desenvolvimento de oligonucleotídeos maiores. Após a seleção da marca RAPD, o fragmento é clonado e sequenciado, sintetizando-se um par de oligonucleotídeos de aproximadamente 24 pares de bases. Esses oligonucleotídeos SCAR são utilizados para amplificar as regiões específicas do DNA genômico. Marcadores RAPD foram convertidos em marcadores SCAR por Zaccaro et al., (2007) para identificação de *Fusarium subglutinans*, agente causal da mal formação da mangueira, e por Domingues et al., (2006) para identificação do caractere de florescimento precoce em *Eucalyptus grandis*.

A seleção no campo de genótipos resistentes ou tolerantes à murcha do *Fusarium* é um processo trabalhoso e caro (Ho, 1999; Javed et al., 2004), e os resultados necessitam ser confirmados por vários ciclos de seleção. O desenvolvimento de estratégias de seleção rápida, confiáveis e reprodutíveis pode acelerar a seleção e promover conseqüentemente o melhoramento genético dos cultivos (Zambrano, 2007).



#### 4 CHARACTERIZATION OF *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* COLLECTED FROM BANANA PLANTS IN THE STATE OF SANTA CATARINA, BRAZIL

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##### Abstract

This work aimed at verifying the genetic variability of *Foc* isolates through levels of aggressiveness and molecular studies (ISSR, SSR and sequencing of the gene TEF-1 $\alpha$ ). A total of 152 isolates collected from banana producing areas in the North and South regions of State of Santa Catarina, Brazil, were compared. They were collected from plants belonging to the Pome and Cavendish subgroups. Pathogenicity was confirmed for all isolates, and the morphological traits confirmed the fungus identity. Greenhouse studies allowed to classify the isolates into three groups according to their aggressiveness level. The results from ISSR, SSR and DNA sequencing revealed less variation since the majority presented 100% similarity with each other. Among these markers, ISSR+SSR was the most polymorphic, being able to differentiate more individuals and with a larger percentage of dissimilarity. When variability data from several DNA markers and aggressiveness data in the present study are analysed together, we observed that for the first set of data there was not a consistent structure of the isolates according to their host or geographical origin, whereas for the second set of data there was a tendency towards a structure according to origin and host.

**Keywords:** *Musa* spp., Panama disease, DNA sequencing, aggressiveness

## 4.1 INTRODUCTION

Banana is an important export commodity for several agricultural-based economies worldwide. Panama disease, or Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the major constraints to crop production. The fungus colonizes banana cultivars from many subgroups, causing plant death and compromising new crops in infested area, since it produces chlamydospores that enable the fungus to persist in soil in the absence of the host. One of the most effective ways to overcoming the problems caused by the disease is using resistant cultivars (Ploetz 2006; Thangavelu et al. 2012), however the development of resistant or tolerant cultivars is expensive and time-consuming (Javed et al. 2004), since the results must be confirmed by many years of field evaluation. In addition, the evaluation of the genetic diversity of the pathogen is also required for efficient management strategies (Kumar et al. 2006). The pathogenicity of strains of *Foc* is based on their ability to cause disease to a given set of cultivars. Races 1 and 2 that were initially reported, affect cv. Gros Michel (AAA) and cv. Bluggoe (ABB), respectively (Waite and Stover 1960). Race 4, the most destructive one, was reported as affecting Cavendish genotypes (AAA), as well as cvs. Gros Michel and Bluggoe (Stover and Malo 1972; Su et al. 1986).

The State of Santa Catarina, located in the South region of Brazil, occupies the third rank in the national banana production, but the crop suffers with Panama disease. The first attempts to circumvent the problems caused by the disease relied on the replacement of cultivars from the Pome subgroup (AAB) with cultivar of the Cavendish subgroup (AAA), considered resistant to *Foc* race 1. However, the recent increasing of occurrence of Fusarium wilt in Cavendish genotypes has challenged this strategy. A great deal of effort has been devoted to understanding the genetic variability and the pathogenicity of *Foc*, and phenotypical and genotypical data are used in combination. Morphology is usually used to separate *F. oxysporum* from other *Fusarium* species, and this can be achieved through the use of selective culture media and observation of mycelium growth and pigmentation. Moreover, microscopic characteristics of reproductive structures (macroconidia, microconidia), types of chlamydospores and phyalides as well as growth rate are consistent criteria (Nelson et al. 1983). Molecular biology techniques, such as RAPD, SSR, AFLP, ISSR and sequencing of the rDNA-ITS, Translation Elongation Factor 1 $\alpha$  gene (*TEF-1 $\alpha$* ) and IGS regions have also contributed to the understanding of

*Foc* variability (Belabid et al. 2004; Bogale et al. 2005; Groenewald et al. 2006; Kumar et al. 2006; Leong et al. 2009; Leong et al. 2010; O'Donnell et al. 1998; Thangavelu et al. 2012).

Considering the importance of the banana crop for southern Brazil and the evergrowing presence of Panama disease in the area, the present research was carried out to evaluate the genetic diversity of isolates of *Foc* from plants belonging to the Pome and Cavendish banana subgroups, using molecular and morphological methods.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Pathogen isolation and monosporic cultures production

Rhizomes from banana plants showing symptoms of infection by *Foc* were collected in 13 counties in the State of Santa Catarina: Jacinto Machado (JM), Santa Rosa do Sul (SR), Siderópolis (Si) and Criciúma (Cr), located in the South region of the State; and Corupá (Co), Jaraguá do Sul (JS), Luís Alves (LA), Massaranduba (Ma), São João do Itaperiú (SJ), Schroeder (Sc), Garuva (Ga), Guaramirim (Gu) and Balneário Piçarras (BP), located in the North region of the State. A total of 152 *Foc* isolates were recovered from Fusarium wilt symptomatic plants belonging to the Pome and Cavendish subgroups, in both regions of the state. The numbers of samples were as follows: 21 from Pome in the South; 40 from Pome in the North; 20 from Cavendish in the South; and 71 from Cavendish in the North. Rhizome fragments were plated on PDA (potato-dextrose-agar) medium for seven days at 25°C±2 under fluorescent light (7.35 W.m<sup>2</sup>, 12-hr photoperiod). After the identification of the causal agent through observation of the reproductive structures (macroconidia and microconidia), monosporic cultures were initiated from each isolate in PDA medium Gonzales and Mendonza (2010).

### 4.2.2 Morphology, pathogenicity and aggressiveness

Monosporic cultures were transferred to CLA medium (carnation leaf-agar) and incubated for two weeks under the conditions previously described, to induce sporulation (Leslie and Summerell 2006). Colony diameter measurements were taken with a caliper and the reproductive structures were visualized in an optical microscope (40x). Colony colour was observed in PDA medium after a 14-day incubation period. The pathogenicity of the 61 isolates from Pome and the 91 isolates from

Cavendish were tested on 6-month-old micropropagated plants of cultivars Prata Anã (AAB) and Grand Naine (AAA), respectively. The plants were cultivated in 3-liter pots filled with substrate composed of 2,400 g of carbonized rice husk, 585 g of chicken manure, 10.5 g of lime, 3 g of NPK 13-40-13 fertilizer, and 1.5 g of Brexil Top (S, Mg, B, Mn, Mo and Zn micronutrients). Ten disks (5 mm diameter each) of *Foc* colonies grown in PDA medium for seven days were inoculated in an autoclaved mixture of 170 g of sand, 30 g of corn meal and 20 mL of water, and incubated at 25°C for 15 days. The concentration was adjusted to  $10^7$  UFC.g<sup>-1</sup>, and the inoculum was divided in eight aliquots which were seeded in each pot, in eight holes made around the base of the plant. A separate inoculum was prepared for each of the 152 *Foc* samples. The micropropagated plants with the inoculum were kept in a greenhouse and the disease severity was estimated 90 days after inoculation (Carlier et al. 2002). The experiment was conducted in a completely randomized design with four replicates, and the average disease severity was compared using the Scott-Knot test ( $p=0.05$ ). Micropropagated plants inoculated with a mixture of sand, corn meal and water but without *Foc* inoculum served as controls.

#### **4.2.3 DNA isolation, ISSR and SSR amplification, and TEF-1 $\alpha$ Sequencing**

A total of 71 *Foc* isolates were selected, representing the three levels of aggressiveness found in the previous test. The numbers of samples were as follows: 14 from Pome in the South; 14 from Pome in the North; 17 from Cavendish in the South; and 26 from Cavendish in the North.

To obtain mycelial mass for the molecular analyses, monosporic cultures were initiated from each isolate in PDA medium as previously described Gonzales and Mendonza (2010), and the DNA extraction was based on Scott et al. (1993). DNA quality and quantity were measured in a biophotometer at 260 nm and 280 nm.

For the ISSR analyses, a total of 14 primers from the UBC series (817, 834C, 834T, 849C, 849T, 851C, 851T, 862, 864, 866, 868, 881, 887DVD and 891HVH) were used. Each ISSR reaction contained PCR buffer 1X, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of dNTP, 0.4  $\mu$ M of primers, 40 ng of template DNA, 1 U of *Taq* DNA polymerase, and sterile water for a final volume of 30  $\mu$ L. PCR was performed on a Verity thermocycler (Applied Biosystems) with the following programme: initial denaturation for 5 min at 94°C, 35 cycles of 30 sec. at 94°C, 30 sec. at

50°C and 90 sec. at 72°C, followed by a final extension time of 7 min. at 72°C. All reaction were made in duplicates. The products were resolved by electrophoresis in 1.2% agarose gel at 70V for 2:30h, and visualized with ethidium bromide under UV light.

For the SSR analyses, a total of nine primers, described by Bogale et al. (2005), were used. The PCR reactions were carried out in a Verity thermocycler (Applied Biosystems), with the following programme: initial denaturation for 5 min at 94°C, 35 cycles of 30 sec. at 94°C, 30 sec. at the specific temperature for each primer, and 90 sec. at 72°C, followed by a final extension time of 10 min. at 72°C. All reaction were made in duplicates. The PCR products were resolved through capillary electrophoresis in an ABI 3130 Genetic Analyzer (Applied Biosystems) and analyzed using the software GeneMapper 4.0.

The sequencing of the Translation Elongation Factor 1 $\alpha$  gene (TEF-1 $\alpha$ ) utilized the primers EF-1 and EF-2 according to (O'Donnel et al. 1998). The PCR reactions contained PCR buffer 1X, 1.50 mM MgCl<sub>2</sub>, 0.40 mM of each dNTP, 0.4  $\mu$ M of each primer, 2.0 U of *Taq* DNA polymerase, 80 ng of DNA, and sterile water to a final volume of 30  $\mu$ L. PCR was performed on a Verity thermocycler (Applied Biosystems), with the following programme: initial denaturation for 5 min at 95°C, 35 cycles of 30 sec. at 95°C, 30 sec. at 60°C and 60 sec. at 72°C, followed by an additional extension time of 10 min. at 72°C. The amplified DNA was further subjected to a sequencing PCR with the Big Dye (Applied Biosystems) sequencing kit. The PCR reactions contained 2  $\mu$ L of Big Dye, 3  $\mu$ L of Save Money buffer, 0.2  $\mu$ M of each primer (frontal or reverse), 50 ng of DNA, and sterile water to a final volume of 20  $\mu$ L. PCR was performed on a Verity thermocycler (Applied Biosystems), with the following programme: initial denaturation for 5 min at 96°C, 35 cycles of 15 sec. at 96°C, 15 sec. at 56°C and 4 min. at 60°C. The samples were purified with the BigDye XTerminator kit (Applied Biosystems) following the manufacturer protocol. The PCR products that resulted from the sequencing PCR with the forward and reverse primers (two replicates each, total of four sequences per accession) were sequenced in an ABI 3130 Genetic Analyzer (Applied Biosystems). The electropherograms obtained with the software ABI Data Collection were converted in nucleotide sequences using the software Sequencing Analysis Version 5.2.

#### 4.2.4 ISSR, SSR, TEF Sequencing, and Phylogenetic Analysis

The products from both ISSR and SSR were converted in presence/absence (1 and zero, respectively). To test for the combinability of these two data sets, the partition homogeneity test with heuristic search was implemented in PAUP version 4.0b10, using 1,000 replicates and the parsimony optimality criterion. The relation between the individuals was based on the Jaccard coefficient and the UPGMA agglomerative method using the software NTSYS-PC 2.1.

The TEF-1 $\alpha$  sequences were compared with sequences obtained by Fourie et al. (2009), deposited in Genbank (accession numbers FJ664901 to FJ66531), and analyzed through the software Mega 5.0. The same software was used for the grouping of the sequences and the construction of dendrograms. The phylogenies based on maximum likelihood (ML) method based on the Tamura-Nei model.

### 4.3 RESULTS

#### Morphology, pathogenicity and aggressiveness

Colony growth ranged from 4.4 mm.day<sup>-1</sup> to 6.6 mm.day<sup>-1</sup>, Microconidia were unicellular or bicellular, oval in shape, and measured from 5 $\mu$ m to 15 $\mu$ m x 2.5 $\mu$ m to 3.5 $\mu$ m. Macroconidia were falciform and presented three to four cells which measured from 27 $\mu$ m to 40 $\mu$ m x 3.3 $\mu$ m to 5.0 $\mu$ m. Chlamydiospores were globose and measured 7 $\mu$ m to 10 $\mu$ m in diameter (Figure 1). The colour of the colonies varied from white to salmon and purple.



Figure 1- Reproductive strutures of *Fusarium oxysporum* f. sp. *cubense*. A) macroconndidia ande microconidia; B) monophialids; C) chlamydiospores. Bar= 10  $\mu$ m.

The pathogenicity was confirmed for all 152 *Foc* isolates. The 61 isolates from Pome were pathogenic to cultivar Prata Anã, and the 91 isolates from Cavendish were pathogenic to cultivar Grande Naine; control plants did not present symptoms of the disease. Statistical analysis for the aggressiveness of the isolates, based on average disease severity using the Scott-Knot test ( $p=0.05$ ), showed significant differences among the 152 isolates, and resulted in three groups. The first group, named least aggressive, had 6.0% of the isolates; all isolates from this group were from the Cavendish subgroup, 8 from the North region and 1 from the South. The second group, named moderately aggressive, had 16.4% of the isolates; 7 isolates were collected from Pome in the North, and 18 were collected from Cavendish, 15 from the North region and 3 from the South. The third group, named aggressive, had 77.6% of the isolates; it was formed by 54 isolates from Pome (33 from the North region and 21 from the South) and 64 isolates from Cavendish (49 from the North region and 16 from the South).

When the isolates from the two subgroups are analyzed separately (Table 1), it can be observed that 11.5% of the Pome isolates were classified as moderately aggressive and 88.5% as aggressive; none was classified as least aggressive. Isolates collected in the North region were classified as moderately aggressive (17.5%) and aggressive (82.5%), and all isolates collected in the South were classified as aggressive. On the other hand, when only isolates from Cavendish are considered, 10.0% were classified as least aggressive, 20.0% as moderately aggressive, and 70.0% as aggressive; isolates collected in the North region were classified as least aggressive (11.3%), moderately aggressive (21.1%) and aggressive (67.6%), and isolates collected in the South region were classified as least aggressive (5.0%), moderately aggressive (15.0%) and aggressive (80.0%).

Table 1 - Aggressiveness levels of 152 isolates of *Fusarium oxysporum* f. sp. *cubense* originated from Cavendish and Pome banana plants grown in the North and South of Santa Catarina, tested on ‘Prata Anã’ and ‘Grande Naine’ respectively.

HOST AND GEOGRAPHIC ORIGIN OF ISOLATE				
level of aggressiveness <sup>a</sup>	pome <sup>b</sup>		cavendish <sup>b</sup>	
	north	south	north	south
least aggressive	none	none	C04, C05, C06, C45, C55, C68, C69, C107	C104
moderately aggressive	P05, P06, P09, P10, P11, P12, P13	none	C01, C07, C08, C09, C10, C12, C13, C15, C27, C34, C36, C37, C44, C51, C58	C84, C85, C99
aggressive	P01, P03, P04, P07, P08, P14, P17, P21, P22, P23, P24, P25, P29, P30, P31, P32, P33, P34, P35, P36, P59, P60, P61, P62, P63, P64, P65, P66, P67, P68, P69, P70, P71	P37, P38, P39, P40, P41, P42, P43, P44, P45, P46, P47, P48, P50, P51, P52, P53, P54, P55, P56, P57, P58	C02, C03, C14, C16, C18, C20, C21, C22, C23, C24, C25, C26, C28, C29, C30, C31, C32, C33, C35, C40, C41, C42, C43, C46, C47, C48, C49, C50, C52, C54, C56, C57, C59, C60, C61, C62, C63, C64, C65, C66, C67, C72, C73, C74, C75, C108, C109, C110	C81, C83, C86, C89, C90, C91, C93, C94, C95, C96, C97, C98, C100, C102, C103, C105

a The average disease severity was compared using the Scott-Knot test ( $p=0.05$ ).

b The pathogenicity of the 61 isolates from Pome and the 91 isolates from Cavendish were tested on 6-month-old micropropagated plants of cultivars Prata Anã (AAB) and Grand Naine (AAA), respectively.

## Molecular Characterization through ISSR and SSR

Fourteen primers from the UBC series were used for the ISSR analyses; seven of those were selected based on their consistent pattern of amplification. A total of 54 bands, varying from 600bp to 3100bp were obtained, and 44 of them were polymorphic. Primers UBC 834C e UBC 862 produced the highest number of polymorphic bands (Table 2).



Table 2- PCR primers, sequence, total number of amplified fragments, and number of polymorphic fragments. .

primer name	primer sequence	amplified fragments	
		total	polymorphic
UBC 834C	AGA GAG AGA GAG AGA GC	12	11 (92%)
UBC 849T	GTG TGT GTG TGT GTG TTA	06	05 (83%)
UBC 862	AGC AGC AGC AGC AGC AGC	13	09 (69%)
UBC 864	ATG ATG ATG ATG ATG ATG	06	06 (100%)
UBC 866	CTC CTC CTC CTC CTC CTC	05	03 (60%)
UBC 868	GAA GAA GAA GAA GAA GAA	08	07 (88%)
UBC 881	GGG TGG GGT GGG GTG	04	03 (75%)
		54	44 (81%)

Six out of the nine SSR primers were selected for their ability to produce consistent banding pattern, however only markers MB02 and MB17 presented polymorphic bands able to differentiate the *Foc* isolates; all others did not present polymorphic bands.

SSR marker MB02 generated a 254bp allele for most of the isolates; the exceptions were isolate S(SR)P52, that presented a 256bp allele, and isolates S(Si)P58 and N(Co)C74, that did not amplify the marker and were considered as having a null allele. SSR marker MB17 generated a 298bp allele for most of the isolates; the exceptions were S(SR)P51, S(Si)P54, S(Si)P57, S(Si)P58 and N(Sc)P59, that did not amplify the marker and were considered as having a null allele.

The partition homogeneity test supported the combination of the SSR and ISSR datasets ( $P=0,296$ ). Cluster analysis with the SSR and ISSR data combined showed that 64 isolates presented 100% similarity with each other (Figure 2); the remaining 7 isolates (9.85%) presented dissimilarities ranging from 2.6% to 4.4%. The isolates with the least dissimilarity (2.6%) were S(SR)P51, S(Si)P54, S(Si)P57 and N(Sc)P59, and the isolates with the largest dissimilarity (4.4%) were N(Co)C75, S(Sr)P52 and S(Si)P58.

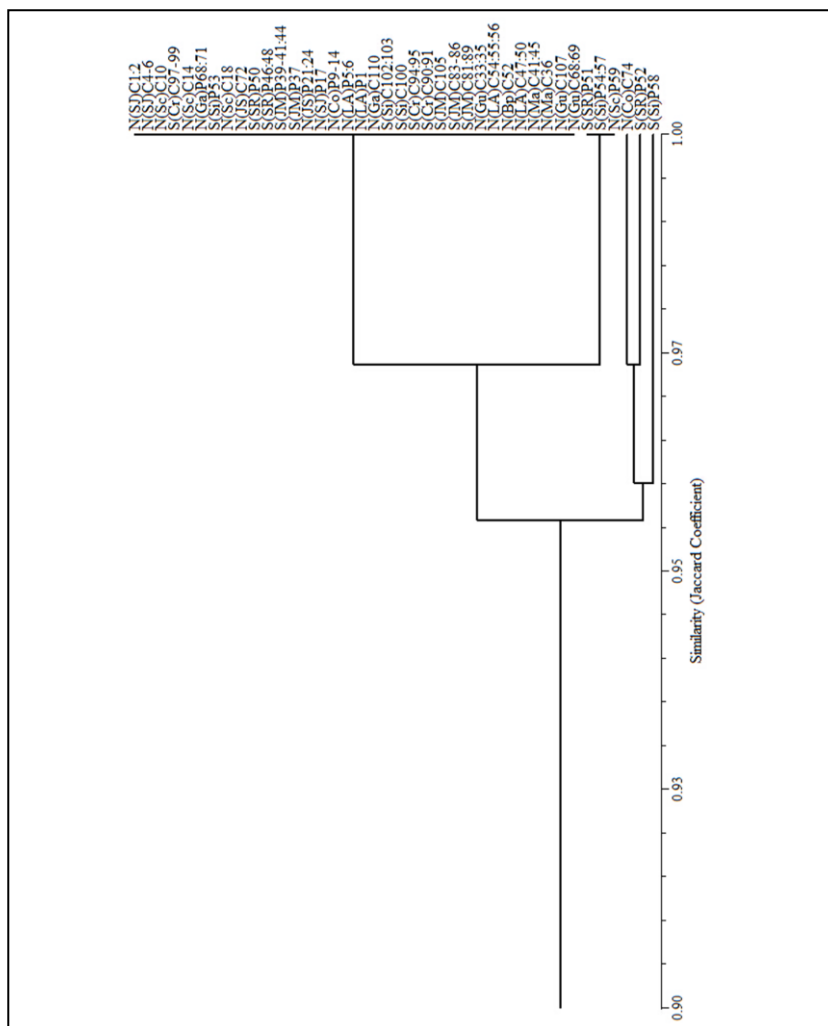


Figure 2- Genetic similarity among 71 isolates of *Fusarium oxysporum* f. sp. *cubense* from banana, using ISSR and SSR data. The similarity was calculated using the Jaccard coefficient, and the dendrogram was constructed using the UPGMA hierarchical agglomerative method. Isolates were collected in 13 counties in the North (N) and South (S) banana producing areas in plantations from the Pome (P) or Cavendish (C) subgroups: Cr = Criciúma; Co = Corupá; Ga = Garuva; Gu = Guaramirim; JM = Jacinto Machado; JS = Jaraguá do Sul; LA = Luis Alves; Ma = Massaranduba; BP = Balneário Piçarras; Sc = Schoreder; Si = Siderópolis; SJ = São João do Itaperiú; e SR = Santa Rosa do

Sul. Hyphen between numbers indicates intervals of isolates.

### **Sequencing of the Translation Elongation Factor (*TEF-1 $\alpha$* ) and phylogenetic analysis**

The PCR products resulting from the amplification using the EF-1 and EF-2 primers had 567bp of good quality to be used for further evaluations. There was no variation in the nucleotide sequences among the 71 isolates from the present work. The phylogenetics analysis carried out with the TEF sequences from our isolates combined with those reported by Fourie et al. (2009). (Genbank accession numbers FJ664901 to FJ66531, total of 49 accessions) plus sequences from other *formae speciales* of *Fusarium oxysporum* (FCC3171\_conglutinans; NRRL\_26178\_melonis; NRRL26602 lycopersici; NRRL 26406 melonis; and CAV337 vasinfectum) and a *Fusarium spp* (NRRL\_22903) is represented in Figure 3. The analysis reveals the existence of two distinct clades, named A and B.

Clade A represents all 71 isolates from the present work plus 33 isolates from Fourie et al. (2009), all belonging to FOC. Within clade A one can find three distinct groups: (i) a larger one formed by our isolates plus 18 isolates from Fourie et al. (2009), all associated with the VCGs 0122, 0120, 0120-15, 01215, 0126, 01210 and 01219; (ii) a group formed by 11 isolates from Fourie, associated to VCGs 0121, 01213 and 01216; and finally (iii) a smaller group, formed by four isolates from Fourie, representing the VCGs 0129 and 01211.

Clade B is composed by 17 isolates from Fourie, belonging to VCGs 01217, 01218, 0123, 01214, 0125, 01212, 0124, 0128 and 01220, plus other *formae speciales* (*melonis*, *lycopersici* and *vasinfectum*), as well as the outlier (*Fusarium sp.*).

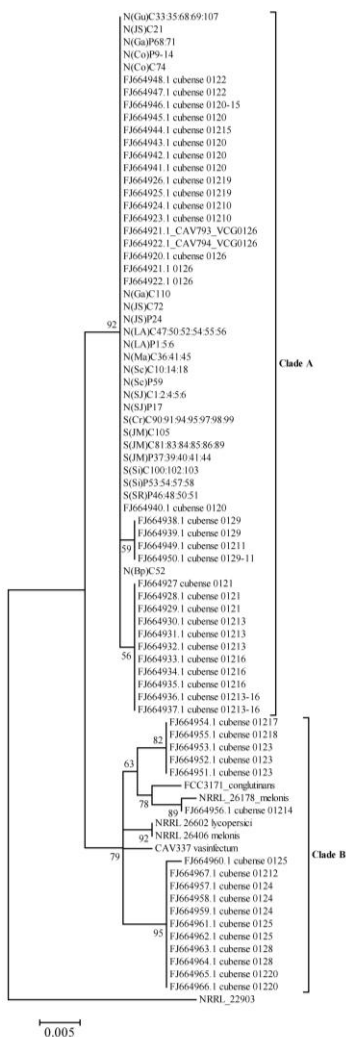


Figure 3- ML phylogenetic tree of *Fusarium oxysporum* f. sp. *cubense* inferred TEF-1α sequence data. The analysis carried out with the TEF sequences from our isolates combined with those reported by Fourie et al. (10) (Genbank accession numbers FJ664901 to FJ66531, total of 49 accessions) plus sequences from other formae speciales of *Fusarium oxysporum*. The two major clades are indicated at their respective branches with A and B. NUMers at nodes indicate bootstrap support (>50%). The tree is rooted with *Fusarium* sp. strain NRRL 22903.

#### 4.4 DISCUSSION

Morphological and molecular characterization of *Foc* is essential for the banana breeding programmes aiming at the selection of resistant plants and as a tool for the integrated management of *Foc*, since the only measure to effectively control the problem is the use of resistant cultivars (Ploetz 2006). The morphological and pathogenicity data collected confirmed the identity of the 152 isolates as *Fusarium oxysporum*, according to the literature (Ploetz 2006; Nelson et al. 1983). The aggressiveness analyses showed a significant variation among the isolates. This variation was also found by other authors when *Foc* isolates from *Heliconia* were studied (Castro et al. 2008).

It was observed that 80.0% of the isolates collected from cultivar Grande Naine in the South region were among those considered aggressive, whereas only 67.6% of the isolates from the same host cultivars in the North were classified as aggressive. In addition, 100% of the isolates collected from cultivar Prata Anã in the South were aggressive, whereas only 82.5% of the isolates collected from the same cultivar in the North were classified in this group.

As observed by the research and extension services for the State of Santa Catarina, Panama disease traditionally occurs in cultivars from the Pome subgroup, but recently its occurrence has increased in cultivars from the Cavendish subgroup. The temperatures in the South area of the State are lower, mainly in the winter (average minimum temperature of 14.2°C, compared to 17.0°C in the North). According to Ploetz et al. (1990) and Viljoen (2002), lower temperatures in the subtropics are conducive to the occurrence of Panama disease in plants from the Cavendish subgroup. Therefore, the prevalence of the disease in those plants may be explained by their higher vulnerability due to the lower temperatures, and not because the pathogen itself is more aggressive (Brake et al. 1995); this could be better understood with experiments with VCG groups. Our data suggest that isolates from the South are more aggressive when inoculated under controlled condition, in greenhouses. This indicates that in South has become a more frequent presence of more aggressive isolates.

The results from genetic diversity by ISSR+SSR and sequencing of the TEF gene showed that few isolates were genetically distant, since the majority presented 100% similarity with each other. Among these markers, ISSR+SSR was the most polymorphic, being able to differentiate more individuals and with a larger percentage of dissimilarity.

In addition, no correlation was found among the origin or the host of the isolate (North or South regions, and Pome or Cavendish subgroups). The Pome isolates used in the present study were also analyzed by Silva et al. (2010) through RAPD and SSR, and likewise no correlation was found. Several other *Foc* genetic variability studies have been carried out in the last years, and the results are quite variable, depending upon the molecular technique utilized and the geographical and host origin of the isolates. Silva-Handlin et al. (1999) studied 14 isolates from the Northeast region of Brazil, collected from cultivars Pacovan, Maçã, Prata and São Tomé; they observed 100% homology among all isolates when the sequencing of the rDNA ITS-5.8S was considered. The PCR-RFLP technique was applied to this same DNA region in several isolates of *Foc* from Malasia and Indonesia, and likewise low variability was observed (Leong et al. 2009). In a study with ISSR carried out by Thangavelu et al. (2012) in India, analyzing 98 isolates from several banana hosts from different genomic groups, six groups were found which were associated to the race of the isolates. Visser et al. (2010), in a study with 45 isolates from several genotypes and geographic origins, found low variability among the isolates when the sequences for the TEF gene were compared.

In the present study, the variability found in the SSR analyses was mainly due to the presence of null alleles for some microsatellites. The occurrence of null alleles in several organisms is mentioned in the literature (Cruz et al. 2009; Santos et al. 2010), and although it may pose some difficulties for the analyses, it should not be ignored, since it reflects a source of genetic variation.

Likewise, the translation elongation factor gene (TEF-1 $\alpha$ ) is widely used for *Fusarium* species identification (Geiser et al. 2004) but also shows a degree of variation within isolates of *Foc* (Fourie et al. 2009; Leong et al. 2010; O' Donnell et al. 1998). In the present study, the application of this technique showed no variation among our isolates, which presented a 100% similarity. Furthermore, when they are compared with the sequences found by Fourie et al. (2009) and deposited in the genbank, it is clear that our isolates are grouped together with isolates from lineages I, II and IV which are associated with the VCGs 01219, 0126, 01210, 01215, 0120, 0115 and 0122. Although we did not carry out analyses for the determination of groups of compatibility with our isolates, it is plausible to hypothesize that the results summarized in our Figure 3 allow the assignment of our isolates to the VCGs which are, in turn, associated with races 1 and ST4. According to the classification made by Fourie, the following

assignment can be made: race 1, isolates 01215 from Nigeria, 0120 from Brazil, and 0120 from Honduras; and race ST4, isolates 0120 from Nigeria, 0115 from Nigeria, 0120 from the Canary Islands, 0122 from the Philippines, and 0120 from South Africa. Therefore, our isolates belong most likely to race 1, although it is known that environmental conditions play an important role in the determination of races, i. e., isolates from one “race” can cause disease in the subtropics but not in the tropics (Su et al. 1986). This is particularly important for VCG 0120: since it causes disease in Cavendish in the subtropics, it would be classified as race 4, but as race 1 because it does not affect plants from the same cultivar under tropical conditions. Therefore, the present classification system is not accurate, and one could consider that all isolates from VCG 0120 belong to the same race, despite the differences in the development of disease under different environmental conditions (Groenewald et al. 2006). It is widely known that there is a great difficulty in standardization of experiments, particularly in the greenhouse, since the ability to cause disease by a particular isolate is influenced by temperature, age of the test plants, method of inoculation and other variables (Correl, 1991).

When variability data from several DNA markers and aggressiveness data in the present study are analysed together, we observed that for the first set of data (ISSR+SSR, and TEF sequencing) there was not a consistent structure of the isolates according to their host or geographical origin, whereas for the second set of data (aggressiveness) there was a tendency towards a structure according to origin and host: isolates from the South area of the State were more aggressive than isolates from the North area, and isolates from Pome were more aggressive than isolates from Cavendish. A possible explanation for the higher aggressiveness of Pome isolates may be given by the fact that they were only tested in plants from the same genomic group, which is known to be more susceptible. However the observation that the isolates from the South region of the State are more aggressive can not be explained by this phenomenon, since they were collected from both Pome and Cavendish cultivars.

It was also observed that there was a wider range of aggressiveness among the isolates from the North region. This may be related to the history of the banana culture in the North and South regions, since in the North there was the gradual replacement of cultivars from the subgroup Pome with cultivars from the subgroup Cavendish, and this higher variability of host plants may have led to an increase in the diversity of the fungus. Furthermore, it was observed that

the seven isolates that differed from the other 64 isolates, when data from ISSR+SSR are considered (Figure 2), belong to the aggressive group.

With the advancements in the scientific basis, new DNA regions are being discovered that may be found to be linked to virulence genes, and it can be postulated that future molecular studies will be based on these more specific regions rather than in random regions, such as is the case with RAPD, SSR and ISSR (Sutherland et al. 2013). Therefore, further studies are necessary to reveal the genetic structure of *Foc* in Santa Catarina, due to the increase in the levels of field infection particularly in areas with cultivars from the subgroup Cavendish.

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## 5 A SCAR MARKER FOR IDENTIFYING SUSCEPTIBILITY TO *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* IN BANANA

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### Abstract

Fusarium wilt (also known as Panama disease) caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is an endemic disease in all banana producing areas. The use of resistant cultivars is the recommended method for the disease control. The development of resistant or tolerant cultivars is expensive and time-consuming since the results must be confirmed by many years of field evaluation. In this situation, DNA markers show a great potential to improve the efficiency and precision of conventional plant breeding. Thus, the objective this work was to develop a SCAR marker able to discriminate between genotypes resistant and susceptible to *Foc* infection. Using genomic DNA from resistant or susceptible genotypes and 78 10-mer arbitrary primers, one RAPD band associated to susceptibility was selected and used to generate the SCAR marker SuscPD-F/SuscPD-R. This marker was validated on 28 banana cultivars which were resistant or susceptible to infection by *Foc*. The results indicated a high degree of specificity of the marker, which was able to discriminate between the two contrasting groups (resistant or susceptible). The discriminatory power of the new marker was 93%.

**Keywords:** Panama disease, molecular marker, diagnostic, *Musa*, genetic improvement.

## 5.1 INTRODUCTION

Panama disease, or Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the major constraints for banana crop production worldwide. The fungus infects cultivars from many subgroups, causing plant death and compromising new crops in infested areas. One of the most effective ways to overcoming the problems caused by the disease is using cultivars that are immune or less affected by the disease (Ploetz 2006; Thangavelu et al., 2012). However, the development of resistant or tolerant cultivars is expensive and time-consuming since the results must be confirmed by many years of field evaluation (Javed et al., 2004).

The fundamental basis of plant breeding is the selection of specific plants with desirable traits, in the present case resistance to infection by *Foc*. DNA markers have a great potential to improve the efficiency and precision of conventional plant breeding and/or clonal selection via marker-assisted selection (Collard and Mackill 2008), in which the selection of the desired individuals is aided by the use of molecular markers. The development of molecular techniques allows the determination of markers linked to certain conditions of economic importance and constitutes a strategy for fast, reliable and reproducible selection, accelerating the improvement through knowledge, in early stages, of the reaction to a given characteristic and its interaction with the genotype (Zambrano et al., 2007). Among the PCR-based markers most widely used in molecular genetic studies, SCARs (sequence characterized amplified regions) have the advantage of being less sensitive to the conditions of a standard PCR due to its primer size when compared to RAPD and hence are more specific and reproducible (Joshi and Chavan 2012). Using the technique of random amplified polymorphic DNA (RAPD), Javed et al. (2004) found that three primers showed banding patterns specific to resistant or susceptible seedlings of *Musa acuminata* ssp. *malaccensis*. Although the authors did not attempt to develop a SCAR marker, they were able to show the great potential of the wild *Musa acuminata* ssp. *malaccensis* as a source for banana improvement and also for the synthesis of segregating populations for linkage mapping, gene cloning and DNA markers related to *Foc* resistance. Also using the RAPD technique, Zambrano et al. (2007) were able to develop a SCAR marker derived from the primer OPK-03, amplified only in the susceptible clones, but not in the resistant/tolerant ones. Following this line of study, Barth Akagbuo, et al. (2011) worked

with developing markers for Sigatoka leaf spot disease resistance in banana.

Two specific SCAR markers associated with resistance to *Foc* race 4 were developed by Wang et al. (2011); these authors state that the identified SCAR markers can be applied for a rapid quality control of *Foc*-resistant banana plantlets immediately after the *in vitro* micropropagation stage, and facilitate marker-assisted selection of new banana cultivars resistant to the disease. The use of molecular tools has also led the way to identify fusarium-linked genes that are highly valued in banana breeding programs (Swarupa et al., 2014). Considering the importance of the banana crop for the South of Brazil and the evergrowing presence of Panama disease in the area, the present research was carried out to develop a SCAR marker able to discriminate between genotypes resistant and susceptible to infection by *Fusarium oxysporum* f. sp. *cubense*.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Plant material and DNA extraction

A total of 28 banana cultivars from diverse genomic groups were used. The reaction to infection by the causal agent of Panama disease (*Fusarium oxysporum* f. sp. *cubense*; *Foc*) was recorded under natural conditions in the Banana Germplasm Bank of the Itajaí Research Station as well as in several farmers located throughout the State of Santa Catarina, Southern Brazil, which allowed the classification of each cultivar as either resistant or susceptible to the strains of *Fusarium oxysporum* f. sp. *cubense* present in the State. The genomic groups and respective cultivars were as follows: group AA - Ouro; group AAA - Galil 7, Grande Naine, Nanicão, Roxa, São Francisco, São Tomé, SCS452 Corupá, Williams and Zillig; group AAB - Branca, SCS451 Catarina, Farta Velhaco, Figue Pome Naine, Maçã, Mysore, Pacovan, Prata Anã, Terrinha and Verde; group AAAB - BRS FHIA Maravilha, BRS Platina, BRS Tropical, FHIA-18, FHIA-21 and Ouro da Mata; and group ABB - Figo and Figo Cinza. Genomic DNA was extracted from leaf samples using the protocol described by Doyle and Doyle (1990) with some minor modifications. DNA quantity and quality was evaluated in a BioPhotometer Plus (Eppendorf, Germany), and stock solutions at the concentration of 20ng DNA per microliter were prepared.

### 5.2.2 RAPD analyses

For this phase of the project, four out of the 28 cultivars were used: two susceptible cultivars (Figue Pome Naine and Maçã) and two resistant cultivars (Grand Naine and Williams). PCR amplifications were performed for a total of 78 primers from the Operon series OPA, OPB, OPC, OPG, OPAX, OPF, OPP, OPW, OPX, PU1, PU2, R1, R2, R3, 81, 171, 172 e 173. All reactions contained 40ng of DNA, PCR buffer 1X, 2mM of MgCl<sub>2</sub>, 0.2mM of each dNTP, 0.4μM of primer, 1.5U of *Taq* DNA polimerase, in a final volume of 30μL. The reactions were carried out in a Verity thermocycler (Applied Biosystems, USA) with the following program: (i) an initial denaturation step for 5 minute at 94°C, (ii) 40 cycles of 1 minute at 92°C, 1 minute at 35°C and 2 minute at 72°C, and (iii) a final extension step of 5 minute at 72°C. RAPD products were separated on 1.2% agarose gels in TBE buffer for 2 hours, stained with ethidium bromide (5μg.mL<sup>-1</sup>), visualized under UV light and photodocumented in a Doc-Print VX2 equipment (Vilber Lourmat, France). All reactions were carried out with two replicates. The presence or absence of bands in the resistant or susceptible cultivars was recorded.

### 5.2.3 Cloning and sequencing of the RAPD product

A RAPD band associated to susceptibility was excised from the agarose gel and DNA was purified using the PureLink PCR Purification Kit (Applied Biosystems, USA). The purified DNA was cloned into the pCR2.1-TOPO vector, which was used to transform *Escherichia coli* competent cells. All procedures were done according to the manufacturer's instructions. DNA was isolated from positive clones grown in liquid medium using the PureLink Quick Plasmid Miniprep kit. This DNA was amplified with M13 forward and reverse primers with the BigDye Terminator v. 3.1 Cycle Sequencing kit. Before the capillary electrophoresis in a 3130 Genetic Analyzer, the sequencing PCR products were purified using the BigDye Exterminator Purification kit. The consensus-sequences corresponding to the fragment extremities were analyzed with the Mega software (Tamura et al., 2013).

### 5.2.4 SCAR primers design and validation

Good quality sequences were selected and used to generate SCAR primers through the NCBI/Primer-BLAST tool (Altschul et al.



1990). One set of primers considered linked to *Foc*-susceptibility was used with DNA from all 28 banana cultivars, including the four cultivars used to generate the SCAR primers. To confirm the amplifiability of the DNAs, PCR reactions were carried out with the banana actine gene primers Actine1F and Actine1R, using the PCR conditions described by Gayral et al. (2008). To confirm the presence or absence of the diagnostic band, reactions involving the new putative SCAR primers were carried out. Each reaction contained 80ng of DNA, PCR buffer 1X, 2mM of MgCl<sub>2</sub>, 0.2mM of each dNTP, 0.4μM of each primer, 1.5U of *Taq* DNA polymerase, in a final volume of 30μL. The reactions were carried out in a Verity (Applied Biosystems, USA) thermocycler with the following program: (i) an initial denaturation step for 5 minute at 94°C, (ii) 35 cycles of 45 sec. at 94°C, 45 sec. at 60°C and 90 sec. at 72°C, and (iii) a final extension step of 5 minutes at 72°C. PCR products were separated on 1.2% agarose gels in TBE buffers for 2 hours, stained with ethidium bromide (5μg.mL<sup>-1</sup>), visualized under UV light and photodocumented in a Doc-Print VX2 equipment (Vilber Lourmat, France). In addition, six annealing temperatures (54°C, 57°C, 60°C, 63°C, 66°C and 69°C) and two DNA concentrations (80ng and 160ng) were tested in seven cultivars out of the 28 cultivars used in the experiment. The efficiency of the SCAR primers was determined by the percentage of cultivars whose field reaction to the fungus matched the reaction predicted by the marker.

### **5.2.5 Resequencing of the amplified fragments and databases comparisons**

DNA from the two susceptible cultivars from the first phase of the project (Maçã and Figue Pome Naine) was used to verify the identity of the fragment amplified by the SCAR primers. The reactions were carried out in replicates to have enough product. The PCR products were purified with the PureLink PCR Purification kit and were used as template for a sequencing reaction using the BigDye Terminator v. 3.1 Cycle Sequencing kit with the forward and reverse primers applied separately. The sequences were obtained in a 3130 Genetic Analyzer plus the sequences obtained in the previous phases were submitted to the Mega software (Tamura et al., 2013) to obtain the complete sequences, which were then compared with sequences from the *Musa acuminata* ssp. *mallacensis* and *M. balbisiana* genomes deposited in the Banana Genome Hub database (Droc et al. 2013) and published by D'Hont et al.

2012; Davey et al. 2013), as well as with other sequences deposited in the NCBI databases.

### 5.3 RESULTS

#### **Development of a SCAR marker associated with susceptibility**

A total of 876 bands were generated in the RAPD reactions; 395 bands (45%) were monomorphic, and the remaining 481 (55%) were polymorphic for at least one of the four cultivars. In order to locate a suitable band for developing the SCAR marker, the presence or absence of bands was checked against the reported reaction of the cultivars to infection by the strains of *Fusarium oxysporum* f. sp. *cubense* present in the state of Santa Catarina. Primer OPP-12 generated a band with approximately 1,700 bp that was consistently present in the susceptible cultivars and absent in the resistant cultivars (Figure 1). This band was considered a suitable candidate for the development of the SCAR marker, and was therefore selected.

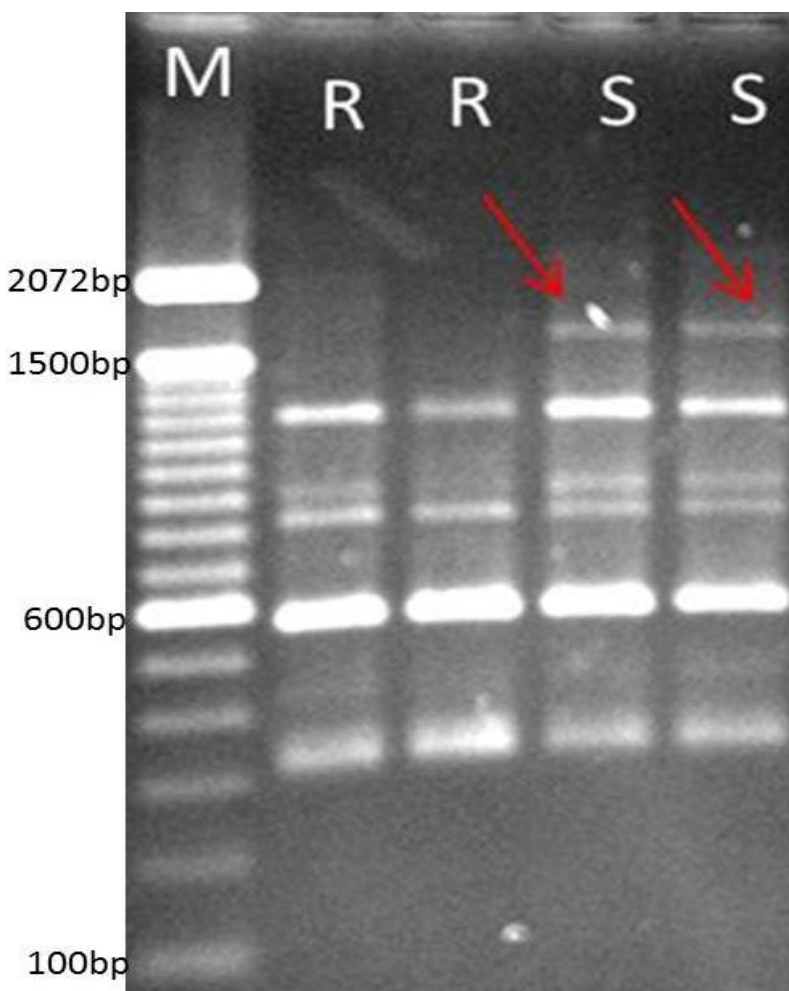


Figure 1 - Identification of RAPD marker associated with *Fusarium oxysporum* f. sp. *cubense* susceptibility in banana. RAPD was performed with DNA from two resistant (R) cultivars (Grand Naine and Williams) and two susceptible (S) cultivars (Maçã and Figue Pome Naine), with a total of 78 arbitrary RAPD primers. A band generated with primer OPP-12 (indicated with an arrow) was successfully converted into a SCAR marker. Lane 1, cultivar Maçã (S); lane 2, cultivar Figue Pome Naine (S); lane 3, cultivar Grande Naine (R); lane 4, cultivar Williams (R). MW = 100 bp molecular weight DNA marker (Invitrogen)

DNA isolated from positive clones was amplified with M13 forward and reverse primers, which produced two sequences with good quality, one generated by the forward primer (642 bp), called left sequence, and one generated by the reverse primer (496 bp), called right sequence. When submitted to the NCBI/Primer-BLAST tool (Altschul et al. 1990), the left sequence generated three sets of forward and reverse primers, and the right sequence generated two sets of forward and reverse primers. These primers were used to amplify DNA from the four test cultivars – two susceptible and two resistant to *Foc*. Initially, five PCR reactions were carried out with the five sets of forward and reverse primers, as generated by the program. Subsequently, six reactions were carried out with the combination of the frontal primers from the left sequence with the reverse primers from the right sequence. Therefore, a total of 11 reactions were carried out.

One combination of primers, hereafter denominated SuscPD-F and SuscPD-R (for Susceptibility to Panama Disease – Frontal/Reverse) was selected based on the consistency of amplification in the two susceptible cultivars and no amplification in the resistant cultivars (Figure 2). The sequences of these primers are: SuscPD-F = GAACCAGAGCCAGGGCATAG, and SuscPD-R = TCTATGCGCCTACCCTCCTT. The annealing temperature ( $T_m$ ), calculated by the program OligoCalc - Oligonucleotide Properties Calculator (Kibbe 2007) was 55.9 °C. In contrast to the reaction with the primer set SuscPD-F and SuscPD-R, the other 10 reactions with the other SCAR primer sets used alone or in combination resulted in no specific amplification from the four cultivars.

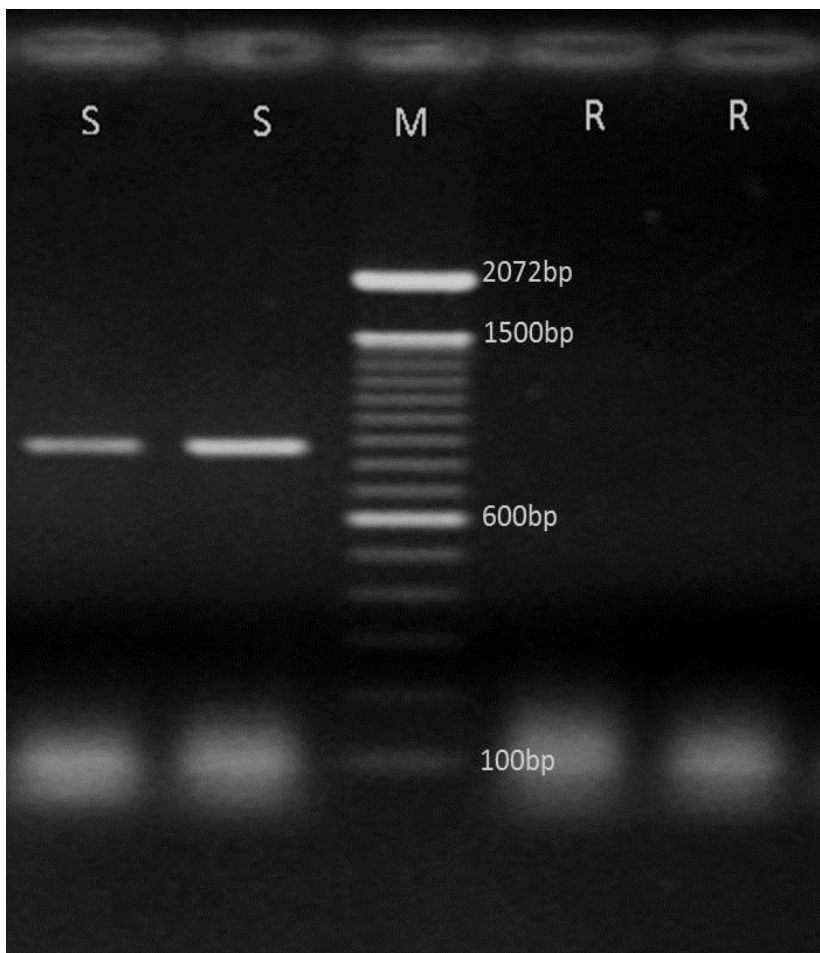


Figure 2 - PCR with SCAR primers SuscPD-F and SuscPD-R used to amplify genomic DNA isolated from leaves of *Foc*-susceptible (S) Maçã and Figue Pome Naine banana cultivars, and *Foc*-resistant (R) Grande Naine and Williams cultivars. Lane 1, cultivar Maçã (S); lane 2, cultivar Figue Pome Naine (S); lane 3, cultivar Grande Naine (R); lane 4, cultivar Williams (R). MW = 100 bp molecular weight DNA marker (Invitrogen)

### **SCAR primer set validation**

The amplificability of the DNA from the 28 banana genotypes (including the four genotypes originally used to generate the SCAR marker) was confirmed by the banana actine gene primers Actine1F and Actine1R, since all DNAs produced the expected band of 420 bp (Figure 3). The results from the PCR reaction with the SCAR primers SuscPD-F and SuscPD-R indicated a high degree of specificity, since they were able to discriminate between the two contrasting groups (resistant or susceptible) in all but two situations (Figure 3; Table 1). The results were the same regardless of the annealing temperature: 54°C, 57°C, 60°C, 63°C, 66°C or 69°C; or the DNA concentration: 80ng or 160ng (data not shown). The discriminatory power of the new marker was 93%.

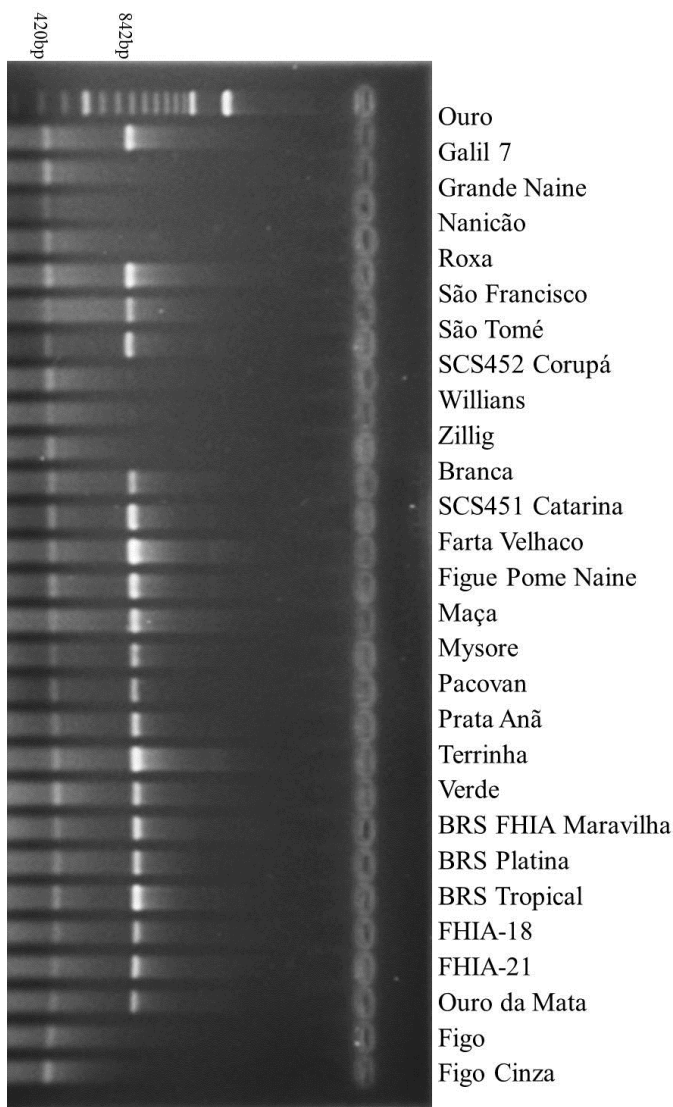


Figure 3 - Multiplex PCR with primers Actine1F and Actine1R used to amplify a 420-bp fragment from the banana actine gene and with SCAR primers SuscPD-F and SuscPD-R used to amplify a 842-bp discriminatory fragment linked to *Foc*-susceptibility. Amplification reactions were performed with genomic DNA isolated from leaves of 28 *Foc*-susceptible and *Foc*-resistant banana cultivars. MW = 100 bp molecular weight DNA marker (Invitrogen)

Table 1 - Reaction of 28 cultivars to natural infection caused by *Fusarium oxysporum* f. sp. *cubense* recorded in the experimental field at the Itajaí Research Station, South Brazil, and reaction predicted from the application of the SCAR marker with primers SuscPD-F and SuscPD-R

Cultivar name	Genomic Group	Reaction to <i>Foc</i>		Cultivar name	Genomic Group	Reaction to <i>Foc</i>	
		Natural in the field	Predicted by the SCAR marker			Natural in the field	Predicted by the SCAR marker
Ouro	AA	S	S	Maçã	AAB	S	S
Galil 7	AAA	R	R	Mysore	AAB	S	S
Grande Naine	AAA	R	R	Pacovan	AAB	S	S
Nanicaão	AAA	R	R	Prata Anã	AAB	S	S
Roxa	AAA	S	S	Terrinha	AAB	R	S
São Francisco	AAA	S	S	Verde	AAB	S	S
São Tomé	AAA	S	S	BRS FHIA Maravilha	AAAB	S	S
SCS452 Corupá	AAA	R	R	BRS Platina	AAAB	S	S
Williams	AAA	R	R	BRS Tropical	AAAB	S	S
Zillig	AAA	R	R	FHIA-18	AAAB	S	S
Branca	AAB	S	S	FHIA-21	AAAB	S	S
SCS451 Catarina	AAB	S	S	Ouro da Mata	AAAB	S	S
Farta Velhaco	AAB	R	S	Figo	ABB	R	R
Figue Pome Naine	AAB	S	S	Figo Cinza	ABB	R	R

Databases comparisons

The alignment of the forward and reverse sequences amplified by the two susceptible cultivars using the algorithms implemented in the Mega software resulted in a fragment of 1672 nucleotides. Comparison of this fragment with the Banana Genome Hub database revealed an identity of 99% similarity with a fragment located in the *Musa acuminata* ssp. *malaccensis* accession Pahang (2n=22) genome. This fragment is located between nucleotides 15,331,281 and 15,332,912. However, no assignment to a particular chromosome or resemblance with known resistance of susceptibility genes was revealed.



## 5.4 DISCUSSION

Panama disease is devastating for the banana crop (Ploetz 2006), and with no doubt the improvement of methods for the selection of plants that carry alleles for resistance or susceptibility to this fungus may greatly help the selection programs. Genetic improvement of banana is complex and lengthy, and the selection of resistant genotypes to the disease is performed in the field. This process spends on average three years to generate results and requires a large experimental area, hand labor and inputs (Javed et al. 2004). The development of a rapid and reliable method capable of detecting resistant genotypes in earlier phases to the field would be of great importance for the banana improvement program against *Foc* (Collard and Mackill 2008).

In the present paper we developed a specific SCAR marker derived from RAPD with a non-specific primer (OPP-12) associated with susceptibility to panama disease, caused by *F. oxysporum* f. sp. *cubense*. SCAR markers have been successfully used in research and plant breeding programs to identify traits of interest (Zaccaro et al. 2007; Zambrano et al. 2007; Joshi and Chavan 2012). By using the DNA from a set of four banana genotypes, two of which were susceptible to infection by the fungus, the SCAR was developed and confirmed, and further confirmation was made with a set of 28 genotypes, 18 of which were susceptible, representing the genomic groups AA, AAA, AAB, AAAB and ABB. Several of them, for example Ouro, Grand Naine, Nanicão, Roxa, São Tomé, Williams, Branca, Catarina, Maçã, Pacovan, Prata Anã, Terrinha, FHIA-18 and Figo are widely used in commercial plantations. The others are also important for small farmers. The amplification of the DNA from these tester plants was in agreement with the reported reaction for 26 out of the 28 genotypes, which indicates a discriminatory power of 93% (Table 1). The SCAR marker development for us is able to discriminate between resistant and susceptible genotypes with a minimal probability of error; since the marker is linked to susceptibility, plants where the amplification of the diagnostic band occurs are said to be susceptible to infection by *Foc* and should therefore be eliminated from the selection programs.

Comparison of this fragment with the Banana Genome Hub database (Droc et al. 2013) revealed an identity of 99% similarity with a fragment located in the *Musa acuminata* ssp. *malaccensis* accession Pahang (2n=22) genome. This fragment is located between nucleotides 15,331,281 and 15,332,912. However, no assignment to a particular

chromosome or resemblance with known resistance of susceptibility genes was revealed. It should be noted that to date only 91% of the assembly was anchored to the 11 *Musa* chromosomes of the Pahang genetic map; further developments may be able to reveal similarities of our sequence to known genes.

The two genotypes whose field reaction to infection differed from the reaction predicted by the SCAR marker are Farta Velhaco (AAB) and Terrinha (AAB); none of them is in commercial use in the South region of Brazil, where the experiment was carried. The SCAR marker gave a positive result, *i. e.*, the susceptible band was amplified even though no infection has been reported so far in these plants under our conditions. It can be hypothesized that the reaction predicted by our SCAR marker is the true reaction and that these cultivars were not yet exposed to conditions favorable for disease development. Even if this is not the case, *i. e.*, the marker has a certain margin of error, the practical outcome of its use will be the erroneous elimination of approximately 7% of the accessions from the genebank, since they would be misclassified as susceptible to *Foc*, even though they were resistant to the disease. It is important to notice that there was no case in which the reaction in the field was “susceptible”, and the reaction predicted by the SCAR marker was “resistant”, implying that no false-resistant accessions would be kept in the genebank.

The fundamental basis of plant improvement is the selection of specific genotypes with desirable traits. DNA markers have a great potential to improve the efficiency and precision of conventional plant breeding and/or clonal selection via marker-assisted selection (Collard and Mackill 2008), in which the selection of the desired individuals is made through the use of some kind of molecular marker. Therefore, the application of the SCAR marker SuscPD-F/SuscPD-R seems to be an efficient and rapid way of selecting genotypes for further tests aiming at the release of new cultivars with resistance to *Foc*. It is expected that once applied to the selection of genotypes generated in the breeding program, this tool will bring considerable gains in terms of time, selection efficiency, as well as cost reduction in area, labor and inputs. Furthermore, the work was developed in a subtropical area, whereas most of the works are carried out in tropical conditions; this makes the information even more important, since subtropical areas are deficient in results and tools in this area.

## 5.5 CONCLUSION

Our results demonstrate that the SCAR marker here reported with the primers SuscPD-F and SuscPD-R is a reliable marker to diagnose *Foc* susceptibility in banana genotypes. The easy of application of the marker, which depends only upon DNA extraction and a simple PCR reaction, can make it a rapid and cost-effective method for the screening of large number of plants, and can help plant breeding programs in Southern Brazil to eliminate *Foc*-susceptible genotypes with a high degree of probability, leaving only the resistant plants for further evaluation.

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## 6 CONCLUSÕES GERAIS

Os resultados do presente estudo permitem concluir que:

- Os estudos relacionados à morfologia comprovaram que, de acordo com os critérios clássicos de definição da espécie, os isolados analisados pertencem a *Fusarium oxysporum* e que, e os testes de patogenicidade confirmaram a identidade de *Fusarium oxysporum* f. sp. *ubense*;
- A análise estatística através do teste de Scott-Knot ( $p = 0,05$ ), para a agressividade dos isolados, mostrou diferenças significativas entre os 152 isolados, e resultou em três grupos distintos: pouco agressivo, moderadamente agressivo e agressivo;
- A diversidade genética por ISSR, SSR e sequenciamento da região TEF-1 $\alpha$  mostrou que alguns isolados foram geneticamente distantes, mas a maioria (88,60%) apresentou 100% de similaridade entre si;
- Os marcadores, ISSR + SSR foram os mais polimórficos;
- Não foi encontrada nenhuma associação entre a origem geográfica do isolado ou a variedade hospedeira infectada com os marcadores estudados;
- A população de *Foc* mostrou-se fenotipicamente diferente com relação aos níveis de agressividade e genotipicamente uniforme, com as ferramentas moleculares utilizadas;
- O marcador SCAR SuscPD-F e SuscPD-R desenvolvido poderá auxiliar o diagnóstico precoce de genótipos de bananeira suscetíveis ao *Foc*.





## 7 CONSIDERAÇÕES FINAIS

Os resultados obtidos neste trabalho poderão servir para apoiar os trabalhos de melhoramento genético da cultura, auxiliando a seleção e a criação de cultivares com maior nível intrínseco de resistência às infecções e melhorias nas técnicas de controle à doença causada pelo fungo. Este trabalho foi desenvolvido em uma área subtropical, enquanto que, as maiorias dos trabalhos vêm sendo realizados em condições tropicais. Este fato reveste estas informações de uma maior importância, uma vez que o subtrópico, zona limítrofe para produção de bananas, tende a demandar técnicas diferentes de manejo do cultivo, decorrentes das adversidades impostas pelo clima da região. Comparando-se o nível de desenvolvimento tecnológico das duas regiões, Tropical e Subtropical, observa-se uma carência de informações técnicas e ferramentas modernas que possibilitem o atendimento das demandas no subtrópico que permitam um incremento tecnológico compatível com a relevância social e econômica regional.

É importante que pesquisas adicionais sejam realizadas uma vez que, com as ferramentas moleculares utilizadas não se encontrou um marcador adequado e associado com os diferentes níveis de agressividade. Novas regiões de DNA ligadas a genes de virulência estão sendo descobertas e, portanto, estudos moleculares futuros deverão ser baseados em regiões mais específicas, que talvez permitam relacioná-las com a origem e virulência do patógeno. Faz-se necessário avançar no conhecimento da estrutura populacional de *Foc* em Santa Catarina, tendo em vista, o aumento das infecções provocadas por *Foc* em áreas plantadas com cultivares do subgrupo Cavendish e a preocupação mundial relativa ao surgimento da raça 4 e suas variantes Tropical 4 (TR4) e Subtropical 4 (ST4) que têm devastado os cultivos comerciais no mundo inteiro.



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